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AF/1654

TRANSMITTAL OF APPEAL BRIEF (Large Entity)

Docket No.
NP4053

In Re Application Of: **Brovelli, et al**

Serial No.	Filing Date	Examiner	Group Art Unit
09/575,307	May 19, 2000	Michael V. Meller	1654

Invention: **ECHINACEA INDUCTION OF PHASE II ENZYMES**

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TO THE COMMISSIONER FOR PATENTS:

Transmitted herewith in triplicate is the Appeal Brief in this application, with respect to the Notice of Appeal filed on **July 18, 2003**

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Amy I. Ahn
Signature

Dated: **September 18, 2003**

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Our File No. NP 4053

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Brovelli et al.

Serial No.: 09/575,307

Filed: May 19, 2000

For: ECHINACEA INDUCTION OF PHASE II ENZYMES

Examiner: Michael V. Meller

Group Art Unit No. 1654

Commissioner for Patents
Alexandria, VA 22313-1450

APPELLANTS' BRIEF UNDER 37 C.F.R. §1.192(a)

This is an appeal from the Final Office Action dated April 18, 2003 rejecting Claims 7-11 and 17-20. By filing, in triplicate, this Appeal Brief in accordance with 37 C.F.R. § 1.192(a), Appellants respectfully request reconsideration by the Board of Patent Appeals and Interferences in the above-identified patent application.

A. REAL PARTY IN INTEREST

The real party in interest is Access Business Group International LLC, located in Ada, Michigan by virtue of an assignment to Amway Corporation executed by the inventors, recorded at Reel/Frame 011158/0657, and an assignment registering the corporate name change from Amway Corporation to Access Business Group International LLC, recorded at Reel/Frame 012958/0620.

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B. RELATED APPEALS AND INTERFERENCES

There are no pending appeals or interferences related to the present appeal.

C. STATUS OF CLAIMS

Claims 7, 8, 10, and 18-20 are currently pending and on appeal. In the Final Office Action, Examiner rejected Claims 7-11 and 17-20. In Appellants' Response after Final, filed July 18, 2003, Appellants consolidated the pending Claims for appeal by canceling Claims 9, 11, and 17 and adding the limitation of the cancelled Claims into independent Claim 7 and Claims 18 and 19 which depend from Claim 7. Claims 1-5 and 12-16 were withdrawn during prosecution. Upon entry of the amendments requested in the Response after Final, Claims 7, 8, 10, and 18-20 remain pending and are on appeal. A copy of all claims as currently pending is included herein as Appendix A.

D. STATUS OF AMENDMENTS

Applicants' amendments to Claims 7, 18, and 19 presented in the Response after Final have yet to be entered.

E. SUMMARY OF THE INVENTION

The invention involved in this appeal relates to methods of inducing the expression of phase II enzyme in a subject in need thereof. In particular, it has been found that administering chloroform-soluble *Echinacea purpurea* extracts induces phase II activity. Phase II enzymes are involved in the detoxification of cancer-causing agents by converting carcinogenic substances into substances that are no longer harmful. Induction of phase II is one mechanism of action correlated with cancer prevention. Unexpectedly, certain fractions of *Echinacea*, particularly the chloroform-soluble fractions, show a greater induction of phase II enzymes than other fractions. Other *Echinacea* fractions tested for phase II induction were as follows: 80% methanol, petroleum ether, chloroform, chloroform (adjusted to ph 2), ethyl acetate, and butanol. In accordance with an aspect of the invention, these chloroform-soluble fractions can be used in a dietary supplement because they are the most potent and can yield the greatest health benefit.

F. ISSUE

Whether it is obvious over Braswell et al. (U.S. Patent No. 6,096,307) in view of Facino et al. (*Direct Characterization of Caffeoyl Esters with Antihyaluronidase Activity in Crude Extracts from Echinacea Angustifolia Roots by Fast Atom Bombardment Tandem Mass Spectrometry*, Farmaco, 1993, 48 (10), 1447-1461) and Intelisano (U. S. Patent No. 6,440,448) to induce the expression of phase II enzyme in a subject in need thereof by administering a chloroform-soluble *Echinacea purpurea* extract as defined by Claims 7, 8, 10 and 18-20.

G. GROUPING OF CLAIMS

Claims 7, 8, 10, and 18-20 stand or fall together.

H. ARGUMENT

Prior to offering Appellants' arguments, it would be useful to review the art of record as applied to the present invention. The primary reference, Braswell, discloses a composition containing an *Echinacea angustifolia* extract, Bromelain, and Lysozyme to control bacterial growth on the tongue and mouth [column 2, lines 11-15]. Examiner appears to have relied on the Braswell reference presumably because of its explanation that "certain *Echinacea* extracts have shown direct anti-cancer activity in vivo" [column 2, lines 61-64]. Facino discloses a method of purifying *Echinacea angustifolia* to obtain a choloroform fraction that has anti-hyaluronidase activity at a certain concentration and acidity [p.1450-1452]. Intellisano discloses a food supplement that contains *Echinacea* as an antioxidant and suggests that *Echinacea angustifolia* and *Echinacea purpurea* are interchangeable.

1. IT IS NOT OBVIOUS UNDER 35 USC §103 TO INDUCE THE EXPRESSION OF PHASE II ENZYMES IN A SUBJECT IN NEED THEREOF BY ADMINISTERING A CHLOROFORM-SOLUBLE *ECHINACEA PURPUREA* EXTRACT.

   a. The Art Of Record Is Insufficient To Support A *Prima Facie* Case Of Obviousness.

Examiner's position on the Claims presented in this Appeal is summarized by the following quotation in the Final Office Action: "Fact is, Applicant is treating cancer. Braswell makes it very clear that *Echinacea* extracts are known to treat cancer, see col. 2 and 3. Thus, it clearly would have been well within the purview of the skilled artisan to carry out the claimed method" [Final Office Action, dated April 18, 2003, page 2]. However, as outlined in the following paragraphs, induction of phase II enzymes relates to chemoprevention [page 1, lines 3-4]. In relying on Braswell, Examiner fails to distinguish cancer treatment from the chemopreventive activity of phase II enzymes and apparently makes the mistaken assumption that cancer treatment necessarily involves induction of phase II enzymes. It is Appellants' position that Examiner has not established a proper *prima facie* case of obviousness because (1) there is no effective motivation or suggestion in the art of record that chloroform-soluble fractions of *Echinacea purpurea* extracts could or should be tried to induce the expression of phase II enzymes; (2) no reasonable expectation of success that the combined teachings of the art of record would successfully induce the expression of phase II enzymes; and (3) the art of record does not teach or suggest all the claim limitations, namely, inducing phase II enzymes with chloroform-soluble fractions of *Echinacea purpurea*. MPEP §2142 states:

> To establish a prima facie case of obviousness, three basic criteria must be met. First, there must be some suggestion or motivation, whether in the references themselves or in the knowledge generally available to one of ordinary skill in the art to modify the reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations. The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art, and not based on applicant's disclosure.

A *prima facie* case has not been properly established because there is no motivation or suggestion to substitute Facino's *Echinacea angustifolia* for Intellisanos' *Echinacea purpurea* and use the resulting composition for treating cancer as taught by Braswell much less use the resulting composition to induce phase II enzymes. To re-summarize, Facino discloses the use of *Echinacea angustifolia* for anti-hyaluronidase. If the *Echinacea* species were interchangeable such that *Echinacea purpurea* were indeed useful to inhibit hyaluronidase, there still is no motivation or suggestion to use *Echinacea purpurea* for treating cancer much less for inhibiting phase II activity because hyaluronidase and phase II

enzymes have different functions and mechanisms. Hyaluronidase is thought to be involved in the migration of cells and plays a key role in the development of inflammatory diseases by depolymerizing or hydrolyzing hyaluronic acid [Facino, p. 1448]. On the other hand, a phase II enzyme, such as quinone reductase, is involved in cell detoxification by reducing oxidized quinone using NAD(P)H. Phase II enzymes also include NAD(P)H, glutathione S-transferases, and UDP-glucuronosyltransferases. Hyaluronidase is also not a Phase II enzyme.

Further, there is no reasonable expectation of success to interchange Facino's *Echinacea angustifolia* for Intellisano's *Echinacea purpurea* and expect that Braswell's cancer treatment or Appellants' phase II induction activity is obtainable. At most, the combination of references makes Appellants' invention no more than "obvious to try". The law is emphatic that "obvious to try" is not the test of obviousness under 35 USC §103. American Hospital Supply Corp. v. Travenol Labs., Inc., 223 USPQ 577, 582 (Fed. Cir. 1984). The Federal Circuit has explained the proper test:

> The consistent criterion for determination of obviousness is whether the prior art would have suggested to one of ordinary skill in the art that this process should be carried out and would have a reasonable likelihood of success, viewed in light of the prior art. Both the suggestion and the expectation of success must be found in the prior art, not in the applicant's disclosure. See In re Dow Chemical Co., 5 USPQ2d 1529, 1531 (fed. Cir. 1988); Amgen Inc v. Chugai Pharmaceutical Co. Ltd., 18 USPQ2d 1016, 1022-23 (Fed. Cir.), cert. denied, 502 U.S. 856 (1991).

The admonition that "obvious to try" is not the standard under §103 has been directed mainly at two kinds of errors. These errors were recited in In re O'Farrell, 7 USPQ2d 1673, 1681 (Fed. Cir. 1988):

> In some cases, what would have been "obvious to try" would have been to vary all parameters or try each of numerous possible choices until one possibly arrives at a successful result, whether the prior art gave either no indication of which parameters were critical or no direction as to which of many possible choices is likely to be successful...In other words, what was "obvious to try" was to explore a new technology or general approach that seemed to be a promising field of experimentation, where the prior art gave only general guidance as to the particular form of the claimed invention or how to achieve it.

Like the many possible choices in O'Farrell, the present case includes prior art that gives no direction as to which of many possible *Echinacea* fractions and variables such as acidities, species, and concentrations is likely to be successful in inducing phase II activity.

The primary reference, Braswell, speaks about *Echinacea*'s uses in generalizations. Of several uses of *Echinacea* that Braswell discloses, Braswell makes one mention that, "...it is believed that *Echinacea* may also provide some effects against tumors. Certain extracts of *Echinacea* root have shown direct anti-cancer activity in vivo" [column 2, lines 62-63]. One of ordinary skill in the art reading Braswell, without hindsight reconstruction, would not know which of several *Echinacea* fractions to explore first for cancer treatment much less phase II induction. In addition, it is known by one of ordinary skill in the art that the activity of a plant based compound changes when any one variable is altered. In fact, the data in support of anti-hyaluronidase activity in Facino and the data in support of Appellant's phase II activity demonstrate that different results are achieved for different *Echinacea* species. While Facino's chloroform fraction (at a neutral ph) of *Echinacea angustifolia* shows less hyaluronidase inhibition than the ethylacetate fraction [Facino, page 1451, paragraph 4 and page 1452, Fig. 1], Appellants' chloroform fraction (at an unadjusted ph and an acidic ph) of *Echinacea purpurea* provides more phase II induction than the ethylacetate fraction [Appellants' application, Fig. 2]. The figures supporting this data are attached hereto as Exhibit B. Because several variables can alter the ability of *Echinacea* to induce phase II enzymes and because none of the cited art motivates or suggests that chloroform-soluble fractions of *Echinacea purpurea* are useful in phase II induction, there is no reasonable expectation of success. The combination of the cited art make it, at best, "obvious to try" the claimed method but fail to make the claimed method for inducing phase II with chloroform-soluble fractions of *Echinacea purpurea* obvious without hindsight reconstruction.

Moreover, a *prima facie* case has not been properly established because all the claimed limitations are not met even if all the cited references are properly combined. See MPEP §2143.03. In particular, none of the cited references teach or suggest the specific method of inducing phase II by administering chloroform-soluble fractions of *Echinacea purpurea*. Examiner's reliance on Braswell's statement that, "Certain extracts of *Echinacea* root have shown direct anti-cancer activity in vivo" falls short of suggesting or teaching that chloroform-soluble *Echinacea purpurea* extract has phase II enzyme inducing capability. Unlike Braswell, Appellants' invention is directed to inducing phase II enzyme, which relates to cancer prevention. Braswell's one mention of "anti-cancer activity" is arguably directed to cancer treatment because the preceding sentence in Braswell states, "...it is believed that

Echinacea may also provide some effects against tumors." Phase II induction is not a mechanism of cancer treatment. Nevertheless, "anti-cancer activity" cannot refer to phase II induction because, as of Appellant's filing date, there were numerous articles indicating that there are several mechanism of action, some unknown, for cancer treatment and chemoprevention. These articles are attached hereto as Exhibit C.

More importantly, the prior art indicated that there were mechanisms of action for chemoprevention with natural compounds aside from phase II induction. These prior art references are attached hereto as Exhibits D through G. Exhibit D discloses that tea polyphenols and green tea have the ability to inhibit growth of cancer cells; also teaches the significance of reducing tumor necrosis factor alpha (TNF alpha) gene expression in cells and TNF alpha release from cells as essential activities for cancer prevention. Exhibit E discloses that Ras proteins serve as central connectors between signals generated at the plasma membrane and nuclear effectors; and disrupting the Ras signaling pathway could have significant potential as a cancer chemopreventive strategy. Exhibit F teaches that gap junction intercellular communication activity can be an effective tool for screening natural products with possible cancer chemopreventive effects. Exhibit G states that inhibitors of protein kinase C may play an important role in the prevention and treatment of cancer. In view of these teachings, phase II induction cannot be suggested or taught by reference to Braswell's "anti-cancer activity" or in combination with the disclosures in Facino and Intelisano. Accordingly, none of the cited references teach or suggest all the claimed limitations to establish a *prima facie* case of obviousness.

b. Appellants' Invention Provides Surprising Results.

Even if the references are properly combined, Appellants believe that the claimed invention is not obvious over the cited art because Appellants' invention provides surprising results as outlined throughout Appellants' specification. MPEP §2144.09 states that, "A *prima facie* case of obviousness based on structural similarity is rebuttable by proof that the claimed compounds possess unexpectedly advantageous or superior properties." Appellants have found that the chloroform-soluble fraction of *Echinacea purpurea* performs surprisingly better than other *Echinacea purpurea* fractions in inducing phase II activity. The phase II inducing activity of the chloroform-soluble fraction is greater than one would expect given

the prior art teachings. A greater than expected result is an evidentiary factor pertinent to the legal conclusion of obviousness of the claims at issue. See MPEP §716.02(a); In re Corkill, 226 USPQ 1005 (Fed. Cir. 1985). As explained previously, Facino's chloroform fraction of *Echinacea angustifolia* shows inferior hyaluronidase inhibition when compared with the ethylacetate fraction [page 1451, paragraph 4 and page 1452, Fig. 1]. As such, if one accepted Examiner's position that the combination of Braswell, Facino, and Intelisano suggests or teaches that *Echinacea purpurea* and *Echinacea angustifolia* were interchangeable for hyaluronidase inhibition as well as phase II enzyme induction, one of ordinary skill in the art would expect that the Appellants' chloroform fraction of *Echinacea purpurea* would result in less phase II activity than Appellants' ethylacetate fraction. However, Appellants' chloroform fraction provides surprisingly superior phase II induction results than the ethylacetate fraction as shown in Fig. 2 of Appellants' application [Exhibit B].

Furthermore, Appellants' claimed method provides surprising results because the level of enzyme activity in the chloroform fractions is significantly higher than other fractions. In particular, the enzyme activity in the root chloroform fraction was 35% higher than the root methanol fraction; the aerial chloroform fraction was 87% higher than the more polar methanol fraction [page 5, lines 15-20]. At a concentration of 0.09mg/ml of *Echinacea purpurea* extract, the root chloroform fraction had 1.86 times the quinone reductase activity of the untreated control [page 9, lines 26-28]. In view of these surprising results, Appellants believe that the claimed method of inducing phase II enzyme by administering a chloroform fraction of *Echinacea purpurea* is not obvious and is patentable over the prior art.

2. CONCLUSION

Based on any one of the arguments set forth above, Appellants respectfully submit that the inventions defined in appealed Claims 7, 8, 10, and 18-20 are not obvious over Braswell in view of Facino and Intelisano. Accordingly, reversal of all grounds of rejection is respectfully requested.

Respectfully submitted,

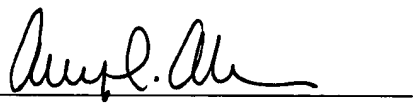

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EXHIBIT A

Claims Currently Pending

7. A method of inducing the expression of a phase II enzyme in a subject in need thereof comprising administering to the subject a chloroform-soluble *Echinacea purpurea* extract.

8. The method of claim 7 wherein the *Echinacea* extract is extracted from the *Echinacea* roots.

9. The method of claim 7 wherein the *Echinacea* extract is extracted from *Echinacea* aerial parts.

18. The method of claim 7 wherein the chloroform-soluble *Echinacea* extract is an effective amount to induce phase II enzyme expression.

19. The method of claim 7 wherein the chloroform-soluble *Echinacea* extract is about 0.09 mg/ml.

20. The method of claim 8 wherein the phase II enzyme has a quinone reductase activity of about 1.86 at 610 nm.

EXHIBIT B

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Quinone Reductase Activity of *Echinacea purpurea* Root and Aerial Part Fractions

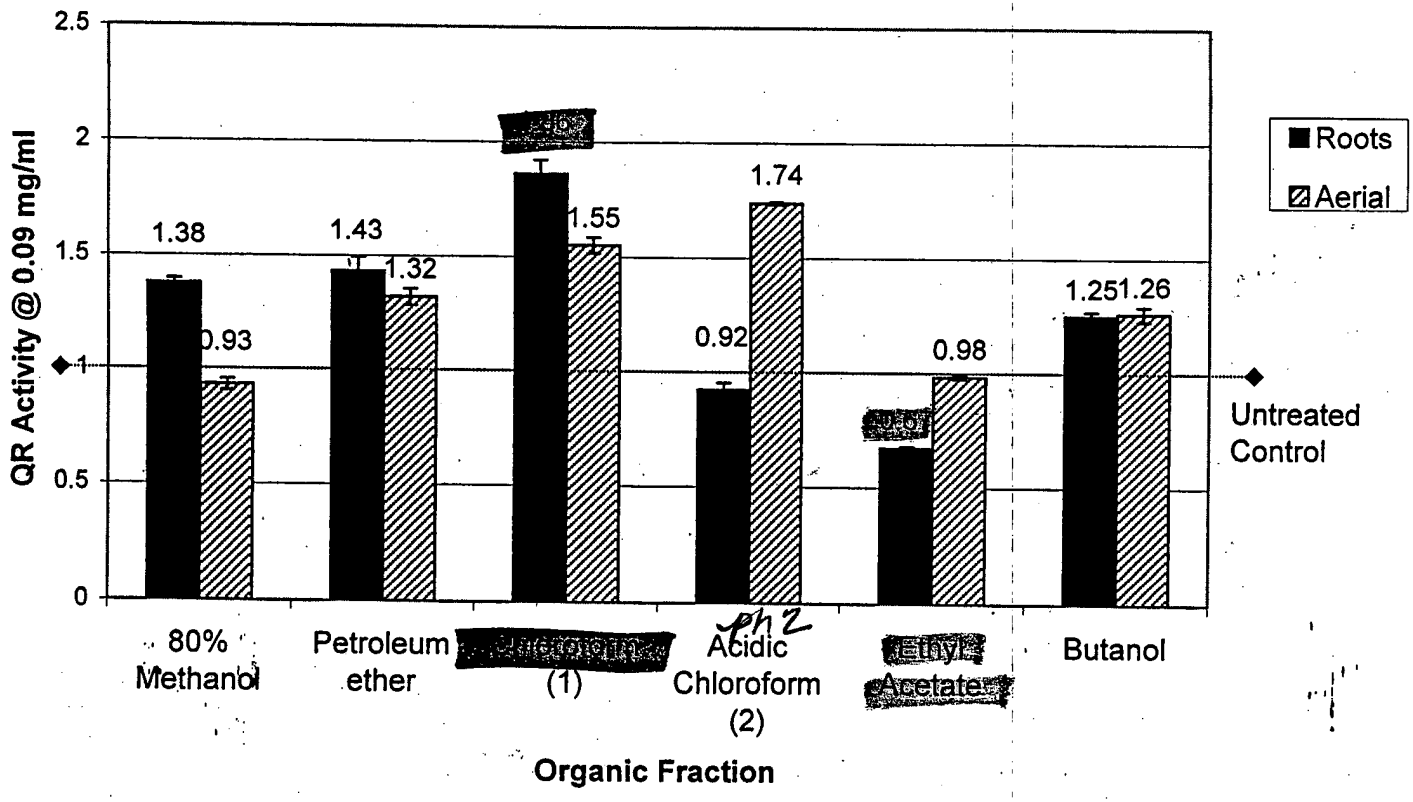

FIGURE 2

Direct Characterization of Caffeoyl Esters with Antihyaluronidase Activity in Crude Extracts from Echinacea Angustifolia Roots by Fast Atom Bombardment Tandem Mass Spectrometry, Farmaco, 1993, 48 (10), 1447-1461.

at 9 eV) shows (Fig. 3a) the base peak at m/z 311, consistent with the cleavage of the ester bond and loss of a caffeoyl residue (162 u), and other diagnostic peaks at m/z 293, 179, 149 and 113, to which the following structures could be assigned:

m/z 311 = [M-Caff-H]$^-$
m/z 293 = [M-CaffOH-H]$^-$
m/z 179 = [CaffOH-H]$^-$
m/z 149 = [Tartaric acid-H]$^-$
m/z 113 = [Tartaric acid-2H_2O-H]$^-$ where
Caff = Caffeoyl residue (162 u); CaffOH = Caffeic acid (180 u).

In a daughter ion spectrum obtained at a collision energy of 29 eV (data not shown), the base peak is at m/z 179 and in addition there are a few other ions of low intensity (not > than 20%), not detectable at 9 eV:

m/z 161 = [Caff-H]$^-$
m/z 135 = [CaffOH-CO_2-H]$^-$
which are consistent with a caffeoyl structure.

All these data suggest the structure of chicoric acid, (2,3-O-dicaffeoyltartaric acid), a compound already isolated from and identified

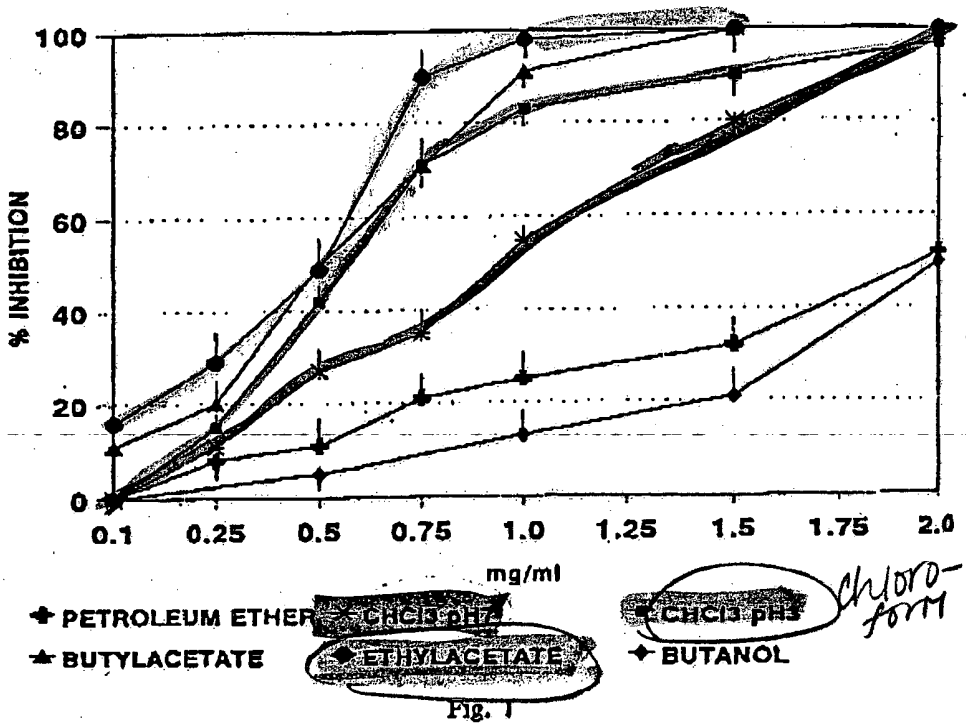

Fig. 1

Antihyaluronidase activity of Echinacea angustifolia *extracts. Values are the means ± S.E. of 5 determinations.*

Material may be protected by copyright law (Title 17, U.S. Code)

EXHIBIT C

☐ Plant-derived anticancer agents.

18/7/6 (Item 6 from file: 155)
DIALOG(R)File 155:MEDLINE(R)
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10837720 97188972 PMID: 9037244
Pezzuto J M
Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago 60612, USA. John.M.Pezzuto@UIC.EDU
Biochemical pharmacology (ENGLAND) Jan 24 1997, 53 (2) p121-33
Natural product drugs play a dominant role in pharmaceutical care. This is especially obvious in the case of antitumor drugs, as exemplified by paclitaxel (Taxol), vincristine (Oncovin), vinorelbine (Navelbine), teniposide (Vumon), and various water-soluble analogs of camptothecin (e.g., Hycamtin). The most efficient method of discovering drugs such as these (i.e. novel chemical prototypes that may function through unique mechanisms of action) is bioactivity-guided fractionation, and it is certain that additional natural product drugs, some of which should be useful for the treatment of humans, remain to be discovered. For the commercial procurement of structurally complex natural product drugs, isolation from plant material may be most practical. With the advent of combinatorial chemistry and high throughput screening, however, even greater progress may now be expected with natural product leads. **While systemic drug therapy, to an appreciable extent based on natural products, has been the mainstay of pharmaceutical care, the logic of disease prevention is overwhelming.** Bearing in mind the pandemic nature of cancer, a proposal is put forth to create a cancer chemoprevention drug formulation for utilization on a widespread basis by the general population. Cancer chemopreventive agents, many of which are natural products, are capable of preventing or inhibiting the process of carcinogenesis. As with other pharmaceutical agents useful for disease prevention, a pharmacoeconomic analysis of a cancer chemopreventive formulation would need to be considered, and the composition of the formulation should improve over time. Nonetheless, implementation should commence immediately. (64 Refs.)

Antitumorigenic effects of limonene and perillyl alcohol against pancreatic and breast cancer.

37/7/8 (Item 8 from file: 155)
DIALOG(R)File 155:MEDLINE(R)
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Crowell P L; Siar Ayoubi A; Burke Y D
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Advances in experimental medicine and biology (UNITED STATES) 1996, 401 p131-6,

Perillyl alcohol is a natural product from cherries and other edible plants. Perillyl alcohol and d-limonene, a closely related dietary monoterpene, have chemotherapeutic activity against pancreatic, mammary, and prostatic tumors. In addition, perillyl alcohol, limonene, and other dietary monoterpenes have chemopreventive activity. **Several mechanisms may account for the antitumorigenic effects of monoterpenes.** For example, many monoterpenes inhibit the post-translational isoprenylation of cell growth-regulatory proteins such as Ras. Perillyl alcohol induces apoptosis without affecting the rate of DNA synthesis in both liver and pancreatic tumor cells. In addition, monoterpene-treated, regressing rat mammary tumors exhibit increased expression of transforming growth factor beta concomitant with tumor remodeling/redifferentiation to a more benign phenotype. Monoterpenes are effective, nontoxic dietary antitumor agents which act through a variety of mechanisms of action and hold promise as a novel class of antitumor drugs for human cancer. (23 Refs.)

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EXHIBIT
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C

PRECLINICAL DRUG DEVELOPMENT PARADIGMS FOR CHEMOPREVENTIVES

Vernon E. Steele, PhD, MPH, Charles W. Boone, PhD, MD, Ronald A. Lubet, PhD, James A. Crowell, PhD, Cathy A. Holmes, PhD, Caroline C. Sigman, PhD, and Gary J. Kelloff, MD

DRUG DEVELOPMENT OVERVIEW

The fundamental goal of the National Cancer Institute's (NCI) chemoprevention drug development program is the identification of safe and effective agents for the prevention of human cancers. By definition, *cancer chemoprevention* refers to the intervention or use of either naturally occurring or synthetic chemical agents to prevent, reverse, or arrest the progression of preneoplastic lesions to invasive cancers.[85] The scientific rationale for conducting chemoprevention trials in humans is based largely on epidemiologic evidence suggesting that dietary components including vitamins and minerals such as β-carotene,[8, 61] vitamin E,[12, 36, 97] calcium,[103] and selenium,[24 37] may be inhibitors of carcinogenesis.[9] Cancer research literature is another important source for identifying potential chemopreventives. In particular, experimental carcinogenesis data from animal studies have shown conclusively that specific chemical sub-

From the Chemoprevention Branch, Division of Cancer Prevention, National Cancer Institute, Bethesda, Maryland (VES, CWB, RAL, JAC, GJK); and CCS Associates, Mountain View, California (CAH, CCS)

stances can prevent both the entire cancer process and the progression of premalignant lesions to invasive cancers.[57, 100] Thus far, several thousand chemical agents have been identified, some of which exist as distinct chemical entities, and others that consist of defined and undefined mixtures. Only through prospective, double-blind, placebo-controlled, clinical chemoprevention trials however, can we determine whether these agents are safe and effective as cancer preventive agents in humans. Key strategies to streamline the selection and prioritization process from preliminary in vitro screening to definitive human clinical trials are important to the NCI's drug discovery and development program, and represent a significant, ongoing, applied-science problem.

In addition to the identification of promising chemopreventive agents, another vital function of the NCI drug development program is the validation and optimization of existing assays and the discovery of new strategic models for assessing chemopreventive activity. This includes standardizing assay protocols using well-established chemopreventive agents and determining various test parameters such as carcinogen dose, treatment time, and dietary considerations. Likewise, efforts to establish more effective screening assays to maximize available resources are being made on an ongoing basis. Overviews of the NCI chemoprevention program have been described in previous publications.[13, 41, 43, 45]

A major strategy in the drug development program is the use of in vitro bioassays for preclinical chemoprevention drug screening. This approach offers a number of important advantages that have facilitated the identification and selection of agents for further in vivo testing. For example, most prescreening and biochemical assays can be conducted within a short time period at a relatively low cost per assay, which not only maximizes the number of agents that can be tested in a single year, but also allows for the rapid determination of biological-activity profiles, mechanisms of action, and the classification of compounds based on compiled data. Additionally, using human cells provides valuable information regarding organ specificity and the cellular toxicity of potential agents. Moreover, these types of assays assist in identifying the appropriate animal model for additional in vivo testing, and preliminary in vitro toxicity data often reduce the need for whole animal testing. Comparisons of structure-activity data and toxicity profiles of candidate agents make possible the identification and selection of a few optimal agents for further testing. Only agents exhibiting low toxicity and high efficacy are chosen for additional in vivo testing in chemoprevention models. Other important criteria such as cost, commercial availability, and dosage formulation also are considered before a promising agent finally is elected for further evaluation in clinical trials. The following sections provide a comprehensive review of preclinical screening assays,

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including mechanistic studies, in vitro bioassays, and animal-efficacy models currently approved and used by the NCI chemoprevention drug development program.

BIOCHEMICAL PRESCREENING ASSAYS

Prior to in vitro efficacy testing, prospective chemopreventive agents are screened in a series of short-term, mechanistic-based assays that measure the inhibition or induction of biochemical processes thought to be involved in the carcinogenic process. Data generated in these assays offer a systematic approach for classifying chemopreventive compounds based on mechanism of action.[42] The precise classification of some agents into a single class is difficult because many of the most promising chemopreventive compounds display a number of mechanistic actions of cancer-blocking activities. Examples of well-known, biological-activity classes include antihormones, anti-inflammatory agents, antioxidants, arachidonic acid (AA) metabolism inhibitors, glutathione (GSH) inducers, ornithine decarboxylase (ODC) inhibitors, and protein kinase C (PKC) inhibitors.

Although elucidation of the precise biological mechanisms involved in preventing human cancers is far from complete, ample evidence to support a number of specific mechanisms in carcinogenesis inhibition has been well documented in the literature.[39, 40, 91, 100, 101] Compilation of mechanistic data from the most effective chemopreventive agents has led to the development of biochemical screening assays that are now currently in use by the NCI to identify new compounds with potential cancer-preventive activity. As illustrated in Table 1, these mechanistic-based assays are divided into three general categories: 1) assays that measure cancer blocking-related activities, 2) antioxidant assays, and 3) biochemical methods that assess antiproliferative- or antiprogression-associated mechanisms. A brief description of enzymatic screening assays and in vitro methodologies used to assess agent-activity profiles in each of these categories is presented subsequently. For a comprehensive review of the molecular mechanisms in carcinogenesis inhibition, refer to the studies in references 39, 91, and 102.

Many agents with cancer-blocking capabilities are compounds that can inhibit carcinogen uptake or binding to DNA, or are effective in preventing the formation or activation of carcinogens. Included in this category of agents are compounds that can enhance electrophile processing by GSH conjugation and tissue-specific oxidative detoxification via induction of phase II enzymes such as glutathione-*S*-transferase (GST) and NAD(P)H-quinone reductase.[7, 99, 107] Examples of chemopreventive agents of this type include vitamins E and C, which work

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Table 1. MECHANISTIC PRESCREENING ASSAYS

Chemopreventive Mechanism	Biochemical Assay
Cancer-Blocking Activities	
Prevent carcinogen binding to DNA	Carcinogen-DNA binding assay
Glutathione reduction	GSH induction
Phase II enzyme induction	GST induction
	NAD(P)H-quinone reductase induction
Antioxidant Activities	
Microsomal enzyme inhibition	GST inhibition
	Peroxisomal enzyme inhibition
Free radical/superoxide inhibition	Cu/Zn superoxide dismutase
	Xanthine oxidase
Arachidonic acid inhibition	Prostaglandin synthase hydroperoxidase
	5-lipoxygenase
Antiproliferative/Antiprogression	
Signal transduction modulation	Tyrosine kinase
Calmodulin inhibition	Cyclic AMP phosphodiesterase
	Protein kinase C
Polyamine synthesis inhibition	ODC inhibition
Polymerase inhibition/induction	Poly(ADP-ribose)polymerase activity
Apoptosis induction	Human epidermal cell assay
Differentiation enhancement	Human epidermal cell assay
Modulation of growth factor and hormonal activity	EGFR/tyrosine kinase
	5α-reductase inhibition
	Aromatase inhibition
Angiogenesis Inhibition	Endonuclease assay
Inhibition of basement membrane degradation	Protease and collagenase inhibition
Prevention of oncogene activation	Ras-farnesylation inhibition
Stimulation of immune response	
Promotion of intracellular communication	

GSH = glutathione; GST = glutathione *S*-transferase; AMP = adenosylmonophosphate; ODC = ornithine decarboxylase; EGFR = epidermal growth factor receptor.

via GSH-enhancing mechanisms,[33] and indole-3-carbinol, an effective chemopreventive in its ability to induce both phase-I and -II enzymes.[84, 92, 106] In vitro assays that allow the assessment of these activities include the GSH-induction assay conducted in Buffalo rat liver cells[79] and measurement of GST[79] and NAD(P)H-quinone reductase[71] induction in the Chang human liver cell line. Inhibition of DNA binding is measured in vitro using immortalized human bronchial epithelial cells (BEAS-2B) that are exposed to the test agent prior to being treated with the carcinogen benzo[*a*]pyrene (B[a]P).[79]

A vast number of the most promising chemopreventive agents possess antioxidant activities and are capable of scavenging activated-oxygen species (i.e., peroxy radicals, singlet oxygen, hydroxyl radical, and superoxide anion). Examples of these include *N*-acetylcysteine and other chemopreventive thiols that are known to sequester reactive elec-

SEP 18 2003 11:35 AM FR CISTI ICIST 613 993 1395 TO 19528512613 P.07/21

trophiles and hydroxyl radicals.[22] Phenolic antioxidants, particularly vitamin E, are effective in scavenging peroxy and superoxide radicals, and singlet oxygen.[29, 33] Oxygen radicals play significant roles in carcinogenesis[34, 81] as evidenced by their ability to induce oxidative damage to DNA, producing strand breaks that ultimately can result in chromosomal rearrangements and deletions.[46] As tumor promoters, free radicals also can modify the transcriptional activation of early response genes, such as c-*fos*, c-*myc*, and c-*jun*, that are associated with cell growth and proliferation.[21] Moreover, once released by inflammatory cells, free radicals and other lipid oxidation products, including those synthesized via AA metabolism (prostaglandins and leukotrienes), can provoke tissue damage, particularly in the colon, and are major contributing factors of inflammatory bowel disease.[47] Assays used to identify antioxidant inhibitory activities include induction of superoxide dismutase (SOD) in mouse epidermal cells and human neutrophils followed by measurement of superoxide generation using chemiluminescence[26, 27, 38] Evaluation of lipid peroxidation inhibition is conducted in mouse or rat liver microsomal preparations,[38, 104] and peroxisomal β-oxidation is quantified in liver supernatants via inhibition of 3-hydroxy-CoA-dehydrogenase.[107] Other assays include measurement of superoxide concentrations, which is determined via reduction of cytochrome C in mouse and human neutrophils.[3, 83] Biochemical methods to assess cyclo-oxygenase 1 (COX-1), COX-2,[20, 28] and 5-lipoxygenase (5-LO) inhibitory activites[15] also are included in this series.

One of the most crucial targets for chemopreventive intervention is the discovery of antiproliferative agents that either interfere or modulate growth regulatory processes required for neoplastic cell proliferation.[70] Blocking signal transduction pathways through inhibition of cyclic AMP, calmodulin, tyrosine kinase, or PKC represents just one of the avenues in which chemical intervention is possible.[32, 41] Another potential strategy is disruption of oncogenic activity mediated by *ras* proteins, which are key regulators of cell growth and differentiation.[44] Inhibition of farnesyl protein transferase (FPT), the enzyme responsible for post-translational modification and activation of *ras* oncogenic activity,[48, 63] allows the identification of agents that can interfere in this prominent mitogenic signaling pathway. Other antiproliferative screens include assays that evaluate inhibition of polymerase activity (poly(ADP)-ribose), an enzyme central to DNA repair,[14] tyrosine kinase,[1, 10, 80] and ornithine decarboxylase (ODC),[52, 68, 96] a key enzyme in polyamine biosynthesis that has been shown to be elevated in tumor tissue and is believed to play a significant role in cell proliferation and malignant transformation.[67]

Another important element of neoplastic cell proliferation involves hormone-mediated growth stimulation. Included in this category are

SEP 18 2003 11:35 AM FR CISTI ICIST 613 993 1395 TO 19528512613 P.08/21

assays that measure inhibition of 5α-reductase,[51] a key enzyme involved in the metabolism of testosterone, and aromatase, a cytochrome P-450-dependent protein that catalyzes the rate-limiting step in estrogen biosynthesis in humans.[30] In addition to these cancer-blocking activities, assays to evaluate other mechanisms of action are being developed and include methods to assess angiogenic inhibitory activity,[65] apoptosis induction,[19, 78] HMG-CoA reductase inhibition,[66, 82] and screening assays to identify agents that effectively block basement-membrane degradation by inhibiting collagenase, hyaluronidase, and other proteases involved in this process.[35, 95] Moreover, development of standard protocols to assess other relevant chemopreventive mechanisms such as induction of terminal cell differentiation,[93] restoration of immune response mechanisms, and effects on intercellular communication[76] are also in progress.

IN VITRO EFFICACY MODELS

In addition to the mechanistic assays previously discussed, systematic evaluation of cancer preventive agents includes screening each compound in a series of short-term, in vitro screens that provide data for selecting and ranking agents for subsequent whole animal testing. Data generated in these assays also may provide insight into potential mechanisms of action. In addition to time and cost considerations, in selecting assays, it is important that: 1) the assays used reflect organ specificity, because cells from different organs may react dissimilarly when exposed to a given test agent, and 2) assays include the use of human primary epithelial cells when possible, because most human cancers are epithelial in origin. Inclusion of primary cells is ideal, because these have relatively intact drug metabolizing systems and still possess a normal genome (i.e., not aneuploid). Epithelial cells are used in all of the five in vitro assays described subsequently. Detailed descriptions of these assays have been published previously[89]; however, a brief overview of each protocol is provided.

The first assay in this series is the rat tracheal epithelial cell assay (RTE), which measures the ability of candidate chemopreventive agents to block B(*a*)P-induced transformation of primary RTE cells.[2, 86, 87] Prior to initiation of the assay, data containing solubility and cytotoxicity are generated for each test compound. Once the appropriate dose levels are determined, tracheal epithelial cells are plated at a uniform density and are incubated for 24 hours with B(a)P in the presence of test compounds. After the 24-hour preincubation period, the cells are rinsed to remove the carcinogen and are allowed to incubate for 30 days in the presence of test agent. Cells subsequently are stained and the number of morphologically transformed foci is determined. Compounds that reduce the

SEP 18 2003 11:36 AM FR CISTI ICIST 613 993 1395 TO 19528512613 P.09/21

transformed foci by 20% or more than the controls without producing signs of cytotoxicity are considered positive.

The second screening assay measures inhibition of anchorage-independent growth in human lung tumor (A427) cells.[49] This assay is an effective method for detecting compounds that block the postinitiation stages of carcinogenesis. Anchorage-independent growth is indicative of the tumorigenic phenotype in most human cells. Similar to the assay described previously, cells are incubated in the presence of test agents for 14 days and then are stained with tetrazolium salt. Positive agents are those that inhibit colony formation by 20% or more.

The third type of in vitro assay measures the ability of test compounds to inhibit the development of carcinogen-induced hyperplastic alveolar nodules (HAN) in mouse mammary gland organ cultures (MMOC).[56, 89] These alveolar lesions are similar in appearance to the precancerous alveolar nodules produced in mouse mammary glands in vivo.[6] Additionally, when grown in hormonally defined culture media, MMOCs undergo the developmental stages of lobulo-alveolar morphogenesis, including differentiation, involution, and glandular regression.[64, 105] Moreover, upon exposure to chemical carcinogens, these organ cultures are capable of oncogenic epithelial transformation.[4, 50] In the present assay, induction of transformation is produced by treating mammary gland cultures with the carcinogen 7,12-dimethylbenz[*a*]thracene (DMBA). According to established protocol, thoracic mammary glands are excised from estradiol- and progesterone-primed mice (BALB/c) and are incubated for 10 to 12 days in culture media containing prolactin, insulin, aldosterone, and hydrocortisone. Following a 12-day developmental period, glandular involution is induced by adding insulin, and the culture is incubated for an additional 14 days. In testing chemopreventive agents in this model, two types of protocols can be followed—a complete carcinogenesis protocol that involves pretreating organ cultures with test compounds prior to DMBA exposure on culture day 3; or an initiation–promotion protocol in which cultured mammary glands are treated with test agents prior to, during, or following exposure to DMBA and TPA (culture days 9 to 24). Once the incubation period is terminated, cells are fixed, stained, and scored for transformation and cytotoxicity determination. Depending on the precise treatment schedule, this assay can distinguish between anti-initiating and antiproliferative activities.

Chemopreventive agents effective in the promotion or progressive stages of carcinogenesis are identified in the fourth in vitro screen, the JB6 epidermal cell assay, which measures the inhibitory capacity of test agents to prevent TPA-induced anchorage dependent growth.[16, 17, 18, 77] After a brief incubation period, cloning efficiency and cytostatic activity of cells treated with TPA and test compounds are determined. An agent

is considered positive if it significantly ($P<0.05$) inhibits growth at one or more nontoxic concentrations.

The last assay in this series is the human foreskin epithelial cell assay, which evaluates the ability of potential chemopreventive agents to inhibit cell growth stimulation induced by the carcinogen propane sultone.[25] Induction of differentiation is assessed by measuring involucrin expression, a precursor molecule of cornified envelope and marker of keratinocyte differentiation. Briefly, primary human foreskin fibroblasts are treated with test compounds in the presence of propane sulfone and are incubated for 5 to 7 days. Percent growth inhibition and cell differentiation are determined as measures of efficacy based on comparison values of cells stimulated with carcinogen alone.

IN VIVO SHORT-TERM SCREENING

Another major emphasis of the NCI drug development program is establishing experimental protocols that allow an assessment of agents that may block or arrest carcinogenesis during the early stages of development. This is particularly relevant because most cancers go through morphologically distinct and identifiable stages over the course of disease progression. Two experimental models that have been investigated for this purpose, which also are representative of major cancers in humans, are the aberrant crypt foci (ACF) assay conducted in rat and mouse colon,[11, 69] and a rat model of mammary gland ductal carcinoma in situ model (DCIS), which is currently under development.[94] In addition to these experimental models, consideration of other short-term systems for chemopreventive drug screening is being evaluated on a continual basis.

The ACF assay is a short-term model that allows the initial assessment and identification of agents that may be effective in preventing colon cancer. ACF are putative preneoplastic lesions consisting of aggregates of single- and multiple-crypt cells that exhibit dysplasia and are thought to be the earliest detectable lesions of colon cancer.[11, 69] Two different protocols have been developed, one that identifies compounds that inhibit initiation and another treatment schedule that evaluates potential chemopreventive agents during the postinitiation phase of colon carcinogenesis. Details of these regimens have been published previously.[88] In the initiation protocol, male F344 rats are given test agents in the diet beginning 1 week prior to the administration of the carcinogen azoxymethane (AOM) and continuing throughout the remainder of the 5-week study. In the second regimen, rats are treated with AOM and 4 weeks later are administered test agent, which is

continued for an additional 4 weeks. Animals are sacrificed and ACF frequency determined by histologic evaluation.

Development of a short-term DCIS mammary carcinogenesis induction protocol for chemoprevention drug screening will provide valuable toxicity and efficacy data for identifying candidate agents for further testing in established mammary carcinogenesis models. According to this protocol,[94] induction of mammary tumorigenesis is initiated in weanling female Sprague-Dawley rats by intraperitoneal injection of the carcinogen, 1-methyl-1-nitrosourea (MNU). Test agents are administered in the diet 1 week after carcinogen administration and continued until termination of the experiment 45 to 50 days later. Mammary glands are excised and processed for histopathologic analysis and classification by standard criteria. Efficacy is measured as percent reduction in the number of DCIS lesions compared with carcinogen controls.

ANIMAL EFFICACY TESTING

Animal efficacy models serve critical functions including establishing organ specificity, providing valuable dose-response data, indicating potential long-term animal toxicity, and providing other key pharmacologic data. In designing in vivo models for chemoprevention drug screening, several important criteria need to be considered. First, the study should be relatively short-term in duration, with induction of cancer in fewer than 6 months. Second, the experimental model should be target specific, evidenced by the production of cancer in the tissue or organ of interest. Ideally, the tumors produced by the inducing agent should reflect similar pathologic characteristics to those found in humans, such as histologic type and hormone dependence. Third, selection of the appropriate model for a given chemopreventive agent should take into account mechanistic data and activity profiles generated from in vitro screens, as well as published data pertinent to the agent's pharmacology and mechanism of action. Depending on the protocol, test agents usually are administered in the diet unless problems with stability or absorption are encountered. During the course of each chemoprevention study, the maximum tolerated dose (MTD) is determined, which is the highest dose level that does not cause a 10% or greater reduction or gain in body weight over a 6-week period. Treatment schedules include administration of test agents only during the carcinogen exposure, only following carcinogen exposure to the end of the experiment, or continuously throughout. Efficacy is determined by the percent inhibition in tumor incidence or multiplicity, or is based on an increase in tumor latency in comparison to carcinogen-treated controls.

Respiratory Tract Cancer Models

Chemopreventive efficacy against respiratory tract cancer can be evaluated in several well-established models. In the hamster tracheal model, the carcinogen MNU is applied directly to the trachea over a 15-week period, which results in the production of squamous cell carcinomas in approximately 40% to 50% of treated animals after 6 months.[60] Test compounds usually are administered over 180 days, beginning 1 week prior to MNU exposure. In the second model, hamsters are administered the carcinogen diethylnitrosamine (DEN) subcutaneously twice a week over a 20-week period, resulting in the formation of lung adenocarcinomas in 40% to 50% and tracheal tumors in 90% to 100% of treated animals.[60] The regimen for test agent administration in this model is comparable to the MNU protocol. A third model known as the *mouse lung adenoma assay* employs the use of strain-A mice, which, following carcinogen exposure, develop lung tumors as early as 6 to 8 weeks, with lung tumor incidences approaching 100% at 12 to 16 weeks of age.[90]

Rat and Mouse Colon Models

Potential inhibitors of colon carcinogenesis are assessed using models developed in both rat (F344) and mouse (CF_1) species.[13, 74] According to established protocols, azoxymethane (AOM) or methylazoxymethanol acetate (MAM) is administered intraperitoneally, which results in colon adenocarcinoma formation within 38 to 40 weeks in either species. AOM is metabolized by the liver to form MAM, the ultimate carcinogen, which is excreted via glucuronide conjugation. In the AOM-induction model, single or dual doses of AOM administered subcutaneously to male F344 rats result in the formation of colon adenocarcinomas and adenomas in approximately 70% of treated animals by 40 weeks.[74] Again, test agents can be administered according to any of the three treatment schedules mentioned previously.

Rat Mammary Gland Models

Chemopreventive efficacy against mammary gland carcinogenesis is assessed routinely by either the MNU- or DMBA-induction models.[31, 53, 57] Both experimental procedures are conducted in female Sprague-Dawley rats and require that the carcinogen be given as a single dose at 50 days of age. In some instances, the carcinogen is administered to older (100 day) animals, which is more representative of the human adult target population.[58] Tumor incidence at 120 days postcarcinogen

treatment is similar, ranging from 80% to 100% in the DMBA protocol, and 75% to 95% in the MNU model; however, the types of tumors produced by the two carcinogens vary. DMBA-induced mammary tumors are usually encapsulated and are predominantly adenomas and fibroadenomas, with some adenocarcinoma development. The majority of tumors produced by MNU, on the contrary, are histologically classified as invasive adenocarcinomas. Chemopreventive activity is based on the percent reduction in tumor incidence or percent increase in tumor latency relative to carcinogen-treated controls.

Mouse Urinary Bladder Model

Bladder tumors induced by the carcinogen *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (OH-BBN) typically are invasive transitional cell carcinomas (TCC) morphologically similar to a type of TCC found in humans.[5, 55, 59] The protocol entails intragastric administration of the carcinogen over an 8-week period to 50-day-old BDF mice (C57BL/6 × DBS/2-F_1) or F344 rats, which typically results in a 40% to 50% tumor incidence at 180-days post-treatment. Treatment schedules for agent administration are those described previously.

Mouse Skin Model

Compounds effective in preventing skin carcinogenesis are identified in a two-stage skin carcinogenesis protocol using DMBA and TPA, which are applied topically to the back skin of SENCAR or CD-1 mice.[54, 98] Both strains of mice are highly susceptible to skin tumor induction. Skin papillomas appear as early as 6 weeks postcarcinogen treatment, eventually progressing to squamous cell carcinomas by 18 weeks.[23] Test agents are generally administered in the diet or, in some experiments, applied topically according to any of the three predefined treatment regimens.

AGENT COMBINATIONS

In addition to single-agent chemopreventive efficacy studies, combinations of agents have been used to measure synergistic or additive effects. Synergistic effects refer to the demonstration of a greater than additive inhibitory potency when a combination of agents is administered together, as opposed to potency data of each agent given alone. This is a particularly useful approach when a promising chemopreven-

tive agent demonstrates significant efficacy but may produce some toxic effects at higher doses. Combinations of agents with either the same or different mechanisms of action may be particularly beneficial in this regard. A few examples of agent combinations producing positive results in experimental animal models include all-*trans*-*N*-(4-hydroxyphenyl)retinamide (4-HPR) and tamoxifen in rat mammary gland,[58, 73] 2-difluoromethylorinithine (DFMO) and piroxicam in rat colon,[72, 75] and 4-HPR and β-carotene in hamster lung.[62] Several of these and other combinations of chemopreventives are undergoing toxicology evaluation or have progressed to phase II clinical trials.

PRECLINICAL TOXICOLOGY AND PHARMACOLOGY

A summary of the preclinical safety studies recommended by the Food and Drug Administration (FDA) for chemoprevention drug screening is illustrated in Table 2 . These studies are generally the same as for other drugs and include the determination of pharmacokinetic parame-

Table 2. PRECLINICAL SAFETY STUDIES FOR INITIATION OF PHASE I AND II CLINICAL TRIALS FOR CHEMOPREVENTIVE INVESTIGATIONAL DRUGS

I. **Required**
- A. Toxicity studies conducted in two species, rodent and nonrodent.
  1. Assess clinical observations, clinical chemistry, hematology, urinalysis, and pathology.
  2. Be of sufficient duration to support the proposed clinical trials (i.e., of equal or preferably greater duration than the proposed clinical trial or up to 6 months in rodents and 12 months in dogs).
  3. Use route of therapeutic administration equivalent to the intended clinical route.
  4. Use drug substances as prepared for clinical trial.
- B. Genotoxic testing in a battery of assays.
- C. Segment I reproductive performance and effect on fertility in rat and Segment II teratology study in rats and rabbits should be conducted as early as possible, prior to large clinical trials of long duration.
- D. Combinations of chemopreventive drugs should be evaluated in at least one study of appropriate duration in the most appropriate species for interactions in pharmacokinetics, toxicity, enzyme effects, or other relevant parameters.

II. **Recommended**
- A. The clinical formulation should be used in all in vivo toxicity studies when possible.
- B. Pharmacokinetics and metabolite profiles should be examined in conjunction with toxicity studies to aid in interpretation of findings and evaluation of relevance to humans.
- C. Pharmacologically guided Phase I clinical starting dose, dosing interval, and dose-escalation strategy should be based upon consideration of concentration-effect relationship shown in preclinical efficacy and toxicity studies.

Adapted from Kelloff GJ, Johnson JR, Crowell JA, et al: Approaches to the development and marketing approval of drugs that prevent cancer. Cancer Epidemiol Biomarkers Prev 4:1–10, 1995; with permission.

ters in acute and subchronic toxicity testing, reproductive studies, and genotoxicity evaluation. Subchronic testing includes repeated daily dosing studies in rodents and nonrodent species as well as an absorption-elimination study in rats. The absorption-elimination data generated in rats provide valuable information in developing methods that are used during drug monitoring in Phase I studies. Dose-concentration profiles generated from preliminary preclinical efficacy testing are useful in approximating a margin of safety along with pharmacokinetic data, and collectively these data further assist in the development of dosing strategies and regimens. Preclinical genotoxicity testing is conducted according to FDA guidelines that recommend the performance of three types of genetic tests. These include: 1) gene mutation studies in *Salmonella typhimurium*, 2) evaluation of genetic mutations in mammalian cells (either mouse lymphoma or Chinese hamster AS52 ovary cells), and 3) in vivo assessment of cytogenetic damage by either the mouse bone marrow micronucleus assay or mouse or rat chromosomal aberration test.

INTERMEDIATE BIOMARKER RESEARCH

Over the last decade, considerable knowledge has been gained in understanding the molecular, cellular, and tissue changes involved in the progression of epithelial neoplasia. An important component of both preclinical and clinical chemoprevention studies is the identification and use of intermediate biomarkers to monitor and validate the degree of cancer progression in epithelial tissue. As part of the drug development program, the NCI has recently expanded its research efforts to evaluate markers in predysplastic and dysplastic tissues as endpoints for assessing chemopreventive agents. This includes evaluating patients with dysplastic lesions such as cervical cancers, actinic keratosis, and oral leukoplakia as well as conducting studies to define potential markers of abnormal cell proliferation, differentiation, and abnormal gene expression. Some of the biomarkers already in use include proliferating cell nuclear antigen (MIB-1), differentiating signals such as blood group antigens, vimentin, and actins.[41] Additionally, efforts to identify biomarkers from animal studies have been initiated in mouse and rat colon, hamster buccal pouch, rat bladder, hamster pancreas, and other experimental carcinogenesis models.

FUTURE DIRECTIONS

The need for continued development and validation of new in vitro screening assays with high predictive value for discovering drugs with

SEP 18 2003 11:40 AM FR CISTI ICIST 613 993 1395 TO 19528512613 P.16/21

human chemopreventive potential is an urgent need and ongoing objective of the drug development program. As our understanding of the molecular and cellular processes involved in carcinogenesis increases (e.g., apoptosis, angiogenesis, signal transduction pathways), experimental assays to measure these and other newly discovered cancer-related mechanisms will continue to be developed. The primary objective is to prevent human cancers; therefore, assays that use human tissues or immortalized cells may be particularly useful in eliminating problems that exist in extrapolating animal data to humans. Moreover, knowledge arising from the Human Genome Project undoubtedly will reveal other relevant mechanisms associated with carcinogenesis. Identification of genes or genetic lesions that may be involved in increasing cancer risk represents a new avenue for chemopreventive intervention and the design of new methodologies to assess the mechanisms associated with this process.

Although most research efforts have focused on cancers of the colon, lung, breast, and bladder, new experimental models and assays for evaluating efficacy in prostate and pancreatic cancer are now being developed. In addition, development of experimental methods to evaluate other cancers with a high prevalence in humans such as brain cancers, leukemia, and non-Hodgkin's lymphoma, will be considered in the near future.

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EXHIBIT D

☐ Farnesyl protein transferase inhibitors as potential cancer chemopreventives.

37/7/6 (Item 6 from file: 155)
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Among the most important targets for chemopreventive intervention and drug development are deregulated signal transduction pathways. Ras proteins serve as central connectors between signals generated at the plasma membrane and nuclear effectors; thus, disrupting the Ras signaling pathway could have significant potential as a cancer chemopreventive strategy. Target organs for Ras-based chemopreventive strategies include those associated with activating ras mutations (e.g., colorectum, pancreas, and lung) and those carrying aberrations in upstream element(s), such as growth factors and their receptors. Ras proteins require posttranslational modification with a farnesyl moiety for both normal and oncogenic activity. Inhibitors of the enzyme that catalyzes this reaction, farnesyl protein transferase (FPT) should, therefore, inhibit Ras-dependent proliferative activity in cancerous and precancerous lesions (J. B. Gibbs et al., Cell, 77: 175-178, 1994). Because growth factor networks are redundant, selective inhibition of signaling pathways activated in precancerous and cancerous cells should be possible. Requirements for Ras farnesylation inhibitors include: specificity for FPT compared with other prenyl transferases; specificity for FPT compared with other farnesyl PPi-utilizing enzymes; ability to specifically inhibit processing of mutant K-ras (the most commonly mutated ras gene in human cancers); high potency; selective activity in intact cells; activity in vivo; and lack of toxicity. Numerous FPT inhibitors have been identified through random screening of natural products and by rational design of analogues of the two substrates, farnesyl PPi and the COOH-terminal CAAX motif of Ras tetrapeptides. A possible testing strategy for developing FPT inhibitors as chemopreventive agents includes the following steps: (a) determine FPT inhibitory activity in vitro; (b) evaluate selectivity (relative to other protein prenyl transferases and FPT-utilizing enzymes); (c) determine inhibition of Ras-mediated effects in intact cells; (d) determine inhibition of Ras-mediated effects in vivo (e.g., in nude mouse tumor xenografts); and (e) determine chemopreventive efficacy in vivo (e.g., in carcinogen-induced A/J mouse lung, rat colon, or hamster pancreas). (187 Refs.)

EXHIBIT F

Effects of Setaria italica on gap junction-mediated intercellular communication for the development of cancer chemopreventive agents

8/7/9 (Item 9 from file: 73)
DIALOG(R)File 73:EMBASE
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Natural Product Sciences (NAT. PROD. SCI.) (South Korea) 1999, 5/2 (88-92) REFS: 19
Inhibition of gap junction-mediated intercellular communication (GJIC) has been considered as an important factor in the tumor promotion phase of carcinogenesis. Recovery effects of natural products on gap junctional intercellular communication are measured by scrape-loading and dye transfer method using Lucifer yellow after administration of phorbol-12-myristate-13- acetate (PMA) on WB F344 cells. Among tested natural products, the hexane fraction and subfractions (F-01 and F-04) of Setaria italica were relatively effective for recovery of GJIC. The hexane fraction of Setaria italica (ECinf 2inf 5, 12.14 mug/ml) and subfractions (F-01: ECinf 5inf 0, 10.74 mug/ml; ECinf 2inf 5, 1.58 mug/ml, F-04: ECinf 5inf 0, 11.03 mug/ml; ECinf 2inf 5, 3.12 mug/ml) revealed dose- dependent recovery effects on GJIC. Our data show GJIC activity measurement by Lucifer yellow spread on cells can be an effective tool for the screening of natural products with possible cancer chemopreventive effects.

EXHIBIT G

Protein kinase C receptor binding assay for the detection of chemopreventive agents from natural products

8/7/20 (Item 20 from file: 73)
DIALOG(R)File 73:EMBASE
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Protein Kinase C (PKC) is generally believed to play a central role in signal transduction, cellular growth control, gene expression, and tumor promotion. And it has been suggested that inhibitors of PKC might play important roles for the prevention and treatment of cancer. In order to investigate the possible inhibitors of PKC from natural products, PKC receptor binding assay was performed using bovine brain particulate as a source of PKC and the amount of (sup 3H)Phorbol 12,13-dibutyrate (PDBu) bound to PKC was measured in the presence of test materials. Total methanol extracts from 100 kinds of natural products were partitioned into 3 fractions (n-hexane, ethyl acetate and aqueous layer) and their binding ability to the regulatory domain of PKC was evaluated. The ethyl acetate fractions of Morus alba (roots, IC$D5inf 0: 156.6 mug/ml), Rehmannia glutinosa (roots, IC$D5inf 0: 134.3 mug/ml), Lysimachia foenum-graecum (roots, IC$D5inf 0: 167.8 mug/ml), Polygonum cuspidata (roots, IC$D5inf 0: 157.3 mug/ ml), Cnidium officinale (aerial parts, IC$D5inf 0: 145.2 mug/ml), and the hexane (IC$D5inf 0: 179.3 mug/ml) and the EtOAc fraction of Symplocarpus nipponicus (roots, IC$D5inf 0: 155.9 mug/ml) showed inhibitory activity of (sup 3H)PDBu binding to PKC.